

Allogeneic hematopoietic stem cell transplantation (HSCT) is commonly used as a treatment for hematological malignancies, immunodeficiencies, and inborn errors of metabolism. HSCT is associated with several complications and risk factors, for example graft-versus-host disease (GVHD), viral infections, relapse, and graft rejection. Infusions of extra lymphocytes from the original stem cell donor can be used as a treatment after transplantation for, e.g., relapse, poor immune reconstitution or secondary graft failure. The term booster applies for infusions of donor cells without conditioning. Secondary graft failure was in this study defined as initial signs of engraftment with subsequent development of bone marrow hyperplasia requiring frequent transfusions beyond day 60 as well as prolonged neutropenia and thrombocytopenia. While high levels of CD3+ cells in grafts can increase the risk for GVHD they can also promote the graft versus leukemia (GVL) effect. In peripheral blood, 95% of T-cells express the $\alpha\beta$ T-cell receptor and the remaining T-cells express the $\gamma\delta$ T-cell receptor, which are not dependent upon human leukocyte antigen (HLA) molecules for activation. As $\alpha\beta$ T-cells are the primary mediators of GVHD, depleting them from the graft should reduce this risk, leaving $\gamma\delta$ T-cells as primary T-cell type in the booster infusion. In this pilot study, five patients transplanted with HLA-matched related and unrelated donors were treated with $\alpha\beta$ T-cell depleted stem cell boosts, with secondary graft failure as primary indication. All patients also suffered from infectious complications prior to the infusion, which is likely to have been associated with the poor graft function and development of secondary graft failure. The $\alpha\beta$ T-cell depletion was performed using a CliniMACS system (Miltenyi Biotech). Analysis of the booster infusion fraction as well as blood samples from the patients was performed; cell characterization by using flow cytometry and evaluation of chimerism as well spectratyping of $\gamma\delta$ -chains by using PCR. Monitoring of white blood cells, platelets, and granulocytes was carried out for 30 days post infusion. The majority of $\gamma\delta$ T-cells in the grafts expressed V δ 2 and/or V δ 9. Median log depletion of $\gamma\delta$ T-cells in the products was 3.72, and the mean yield of $\gamma\delta$ T-cells was 84.4%. Most patients receiving $\alpha\beta$ -depleted stem cell boosts increased their levels of white blood cells, platelets, and/or granulocytes 30 days post infusion. No signs of GVHD or other side effects were detected. A larger pool of patients with longer follow-up time is needed to confirm the data in this study and compare the treatment modality to other therapy options.

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Hypomethylating Agents Induce FOXP3 Negative HLA-G Expressing Immunoregulatory T Cells

Alexandros Spyridonidis¹, Panagiota Stamou², Dimitra Marioli¹, Angeliki Vittoraki³, Vasiliki Goudogianni¹, Efthymia Theofani¹, Argyro Sgourou⁴, Panagiotis Zikos⁵.

¹Bone Marrow Transplantation Unit, Hematology Division, University Hospital of Patras, Rio-Patras, Greece; ²Department of Medicine, University Hospital of Patras, Rio-Patras, Greece; ³National Tissue and Typing Center, Hematology Department, General Hospital of Athens 'G.Gennimatas', Athens, Greece; ⁴School of Science and Technology, Hellenic Open University, Rio-Patras, Greece; ⁵Hematology Division, General Peripheral Hospital of Patras 'Agios Andreas', Patra, Greece

A major caveat of FOXP3+ T regulatory cells in immunotherapy against GVHD is their low numbers in circulation and the lack of specific cell surface markers for efficient purification. DNA methylation plays a key role in the regulation of T-cell effector function and cytokine gene expression,

indicating a promising role of hypomethylating agents in immunomodulation. Recently it was shown that in vitro treatment of conventional T-cells with the hypomethylating agent azacitidine (aza) induced FoxP3 expression and converted CD4⁺CD25⁻ cells into immunosuppressive T-cells, the suppressor function of which is independent of FoxP3 expression (Blood 2010;116:129-139), suggesting that aza induced suppressor function depends on the modification of other hypomethylated genes. Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule, shown to exert immunoregulatory functions, the expression of which is epigenetically regulated. In this study we investigated whether hypomethylating agents (HAs) can induce HLA-G⁺ immunoregulatory T cells. Negative selected T cells from peripheral blood of healthy individuals were stimulated with anti-CD3 plus anti-CD28 coated beads, then treated for 72 hours with aza (0.5-15 mM) in the presence of 50U/ml interleukin-2 (IL-2). Phenotypical characterization of aza treated cells was performed with flow cytometry. The effect of HAs on HLA-G mRNA transcription and methylation of upstream regulatory HLA-G gene DNA sequence was quantitated with Real time PCR and bisulfite pyrosequencing respectively. Hypomethylation-induced HLA-G⁺ T cells, were irradiated and then used as third party cells in suppression assays. In vitro treatment of CD3⁺ T cells with aza induces, dose dependent, de-novo HLA-G expression on stimulated peripheral T cells of healthy individuals. The optimum aza concentration for maximum HLA-G induction with the lowest toxicity in CD3 T cells was determined at 5 mM (surface expressing HLA-G cells: 6.88±3.9%, p=0.0022, fold increase in HLA-G mRNA relevant expression: 18.32±2.33). Methylation analysis of 13CpGs revealed that treatment with HAs caused hypomethylation of the HLA-G upstream-regulatory region followed by mRNA transcription and up-regulation of surface protein expression. Stability assays revealed that HLA-G expression is evident for at least 3 days after removal of hypomethylating agent. In vitro Aza induced HLA-G⁺ cells are immunosuppressive and HLA-G blocking experiments further confirmed that their suppressive function is largely but not exclusively dependent on HLA-G. Phenotypic analysis revealed that aza induced CD4⁺HLA-G⁺ are FoxP3 negative CD4^{low}CD25^{high}. Conclusively, by using hypomethylating agent aza in vitro, we generated a CD4^{low}-FoxP3^{neg} immunoregulatory population, which expresses extracellular HLA-G and therefore can be easily isolated for adoptive immunotherapies.

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Novel Strategy to Enhance NK Cell Activity

David Wald¹, Reshmi Parameswaran¹, Stephen Moreton¹, Marcos J.G. de Lima², Dean Lee³, Paramaeswaran Ramakrishnan¹. ¹Case Western Reserve University, Cleveland, OH; ²University Hospitals Seidman Cancer Center and Case Western, Cleveland, OH; ³Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX

Novel therapeutic approaches are urgently needed for many malignancies such as Acute Myeloid Leukemia (AML). We have developed a new therapeutic strategy based upon NK cell immunotherapy that exhibits high clinical potential based upon cell and animal studies. While the harnessing of NK cells for cellular therapy against malignancies has been a topic of interest for several decades, our approach overcomes a major hurdle of insufficient NK cell cytotoxic activity. We have identified that targeting the kinase GSK3 through pharmacologic and genetic approaches leads to the hyperactivation of human blood derived NK cells and a significant

improvement in efficacy as compared to traditionally used activated NK cells or chemotherapy in our mouse AML model systems. Importantly this GSK3 inhibition can be achieved through a short ex vivo incubation of NK cells with a GSK3 inhibitor paving the way for a rapid implementation into a clinical trial. Utilizing both in vitro studies with AML cell lines (ex. OCI-AML3 and HL-60) and primary human AML cells we observe approximately a 50% increase in efficacy with GSK3 inhibited NK cells as compared to untreated NK cells. Further, we demonstrate significant efficacy of GSK3 inhibited NK cells in a mouse model of circulating human AML. After 4 weekly injections of human NK cells, there is a 50% greater reduction in human AML cells present in the mouse bone marrow with GSK3 inhibited NK cells as compared to vehicle treated NK cells.

Besides efficacy studies, our work has led a model of how GSK3-inhibition enhances NK cell activity as depicted in Figure 1. GSK3 inhibition leads to a dramatic increase in adhesion of NK cells to target cells as demonstrated by a flow cytometric adhesion assay (49% vs 83% after 20 min incubation) as well as live cell imaging. Consistent with the increased adhesion, GSK3 inhibited NK cells as well as target cells (after co-incubation) exhibit increased expression of essential NK cell-target adhesion molecules including L-selectin (on NK cells) and ICAM (on target cells). The induction of ICAM on target cells is due to a marked induction in TNF α production from the NK cells upon incubation with target cells (>7 fold increase in TNF α production). TNF α neutralization impairs the NK activity of the GSK3 inhibited NK cells (~30%) but not vehicle treated cells. Finally, GSK3 inhibition also leads to changes in the NK cells that enhance activity such as increased expression of

granzyme and perforin and secretion of IFN γ . Overall, our work has revealed a novel strategy for NK cell therapy that holds high clinical potential.

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Spinal Cord Injection of Stem Cells for Pediatric ALS

Laura L. Worth¹, John Mark Slopis², Mary Kay Koenig³, Richard E. Champlin⁴, Ian E. McCutcheon⁵. ¹Pediatrics, University of Texas MD Anderson Cancer Center, Houston, TX; ²Neurology, M D Anderson Cancer Center, Houston, TX; ³Neurology, University of TX Health Science Center at Houston, Houston, TX; ⁴Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX; ⁵Neurosurgery, M D Anderson Cancer Center, Houston, TX

Topic Significance & Study Purpose/Background/Rationale:

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that destroys the motor neurons of the brain and spinal cord. With no definitive therapy, most patients die within 3-5 years of diagnosis. Extensive research is underway including the use of stem cells to regenerate damaged neurons. Although typically an adult disease, childhood cases are reported. Our patient began showing symptoms at age 6 and was formally diagnosed with the rare juvenile form of ALS at age 9. At consult, she displayed marked spasticity with loss of fine motor control. She could not walk or communicate verbally and required nocturnal ventilatory support. She was G-tube dependent for nutrition. Because of her young age, she was ineligible for clinical studies. After an ethics consult, FDA, IRB, and financial approvals, we followed Deda's procedure (Cytotherapy 2009;11;18-15).

Methods, Intervention, & Analysis: Autologous, bone marrow cells were harvested from iliac crests and collected by Ficoll density gradient. The following day laminectomy of C1 and partial laminectomy of C2 was performed. A suspension of 6 million cells in 300 microliters was prepared. After a 25 gauge needle was advanced to a depth of 9 – 10 mm into the anterior horn of the cervical spinal cord at a 20 degree angle, 500,000 cells/50uL were injected. Six injections were performed on each side of the midline (12 injections). Each injection was 7 mm apart on the mid-rostral caudal line and 2 mm off midline to each other. Two strips of gel foam each infused with 5 million cells/500uL were placed along the anterior quadrant of the spinal cord prior to dural closure. An additional 5 million cells/500uL were injected into the subarachnoid space. The remainder of the cells were injected intravenously (1.2×10^8 TNC/kg). There were no postoperative complications.

Findings & Interpretation: Peak response was seen at 6 months with 12 month duration: improved phonation, facial movements, swallowing, and upper extremity control. Nocturnal CPAP use was successfully removed. She developed intermittent spasms/clonus with right upper extremity pain. The patient refused additional ventilatory support and with disease progression, died 25 months later of respiratory failure.

Discussion & Implications: Additional studies are needed to evaluate this therapy.

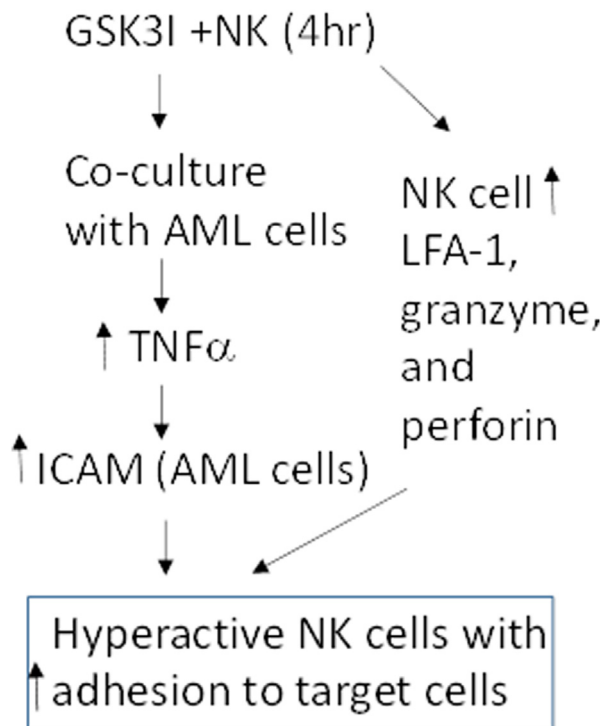


Figure 1. Model of how GSK3 inhibition leads to hyperactive NK cells. GSK3I - GSK3 inhibitor.